status of all citations of U.S. filed applications, as appropriate; and the address for the ATCC (page 7).

Applicant submits that the specifcation is amended herein to update the status of the parent priority applications as well as the address for the ATCC. Applicant was unable to locate any citations to U.S. filed applications that were in need of updating and, therefore, respectfully requests clarification from the Examiner. However, page 4 of the specification is amended herein to indicate more clearly that citations to two U.S. references are to patent documents. Accordingly, Applicant respectfully requests reconsideration and withdrawal of the objection to the specification.

Rejections under 35 U.S.C. § 112, ¶ 2

Claims 1-8 stand rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as their invention. Claims 1-8 are cancelled herein and have been replaced by Claims 20-41. The following remarks are therefore directed to Claims 20-41. Applicant respectfully traverses this rejection.

Regarding Claim 1, the Examiner contends that the phrase "a gene of interest" is unclear because the term "gene" implies a DNA sequence that exists in nature and includes coding and noncoding regions, as well as all regulatory sequences associated with expression. The Examiner suggests that the phrase "a DNA of interest" be recited instead. Applicant submits that this rejection has been rendered moot by the cancellation of the rejected claim and the fact that the newly submitted claims do not recite the rejected language.

The Examiner further contends that the term "exposing" in Claim 1 is unclear and should be amended to "contacting," as "exposing" allegedly could be in the same vicinity without being in contact. Though Claim 1 is cancelled herein, Applicant addresses this rejection here, since the newly submitted claims recite the term "exposing." Applicant disagrees with this rejection and respectfully reminds the Examiner that an applicant "can define in the claims what they regard as their invention essentially in whatever terms they

choose so long as the terms are not used in ways that are contrary to accepted meanings in the art." MPEP § 2173.01. Applicant submits that the term "exposing" is not used in a way that is contrary to its accepted meaning in the art. The terms "exposure," "exposed," and "exposing" are used throughout Applicant's specification (see, *e.g.*, p. 1, lines 16 and 28; p. 2, lines 4 and 17; p. 6, lines 2, 5, and 12; and p. 10, line 4). Thus, when viewed in the light of the specification, the claim term "exposing" satisfies the notice function of § 112, second paragraph, and clearly informs those of ordinary skill in the art of the metes and bounds of the claimed invention.

The Examiner also asserts that the phrase "under conditions which inhibit AIN" in Claim 1 is unclear, since it is allegedly not clear whether the *Agrobacterium*, the plant cell or tissue, or both are under these conditions. This rejection has been rendered moot by the cancellation of the rejected claim and the fact that the newly submitted claims do not contain this alleged defect.

The Examiner further contends that Claim 1 is incomplete because the final step of the method does not produce the desired product. Though Claim 1 is cancelled herein, Applicant addresses this rejection here, since the newly submitted claims are also directed to various methods. Applicant respectfully disagrees with the Examiner. Specifically, a method or process claim may be one of at least three types: a generalized method for accomplishing some specified end, a method of use, and a product-by-process. See, *e.g.*, MPEP § 2112.02. Applicant respectfully submits that, unless a method claim is a product-by-process claim, there is no requirement of which Applicant is aware that the final step of a method must generate a product. Applicant's newly added independent Claim 20 recites "A method for transforming a plant cell or tissue susceptible to *Agrobacterium*-induced necrosis (AIN) with a nucleotide sequence of interest." The claimed method does not require that a product be produced. Applicant therefore respectfully submits that there is no requirement for a final step that produces a product.

Regarding Claim 2, the Examiner contends that the phrase "after heat-shock treatment" is unclear because the object of the "treatment" (*i.e.*, the *Agrobacterium*, the plant cell or tissue, or both) is allegedly unclear. This rejection has been rendered moot by the

cancellation of the rejected claim and the fact that the newly submitted claims do not recite the rejected language.

Regarding Claim 4, the Examiner asserts that the phrase "chemical inhibitor" is unclear and questions whether the "inhibitor" is a chemical or whether the "inhibitor" inhibits a chemical. The Examiner also points out that "phophatase" is misspelled. This rejection has been rendered moot by the cancellation of the rejected claim and the fact that the newly submitted claims do not recite the rejected language.

Regarding Claim 5, the Examiner questions how "ethylene inhibitors" and "ethylene synthesis inhibitors" are different. Though Claim 5 is cancelled herein, Applicant addresses this rejection here, since Claims 27-29 recite "an ethylene inhibitor" and/or "an ethylene synthesis inhibitor." Applicant respectfully directs the Examiner's attention to the specification (p. 3, lines 7-8), where various examples of ethylene inhibitors (*e.g.*, 2,5-norbornadiene, norbornene, silver thiosulfate, and silver nitrate) and ethylene synthesis inhibitors (*e.g.*, aminoethoxyvinylglycine (AVG), cobalt salts, acetyl salicylic acid, and salicylic acid) are provided. Applicant submits that, when viewed in the light of the specification, the distinction between the recited claim terms "ethylene inhibitor" and "ethylene synthesis inhibitor" can be readily appreciated by one of ordinary skill in the art. Thus, the claims satisfy the notice function of § 112, second paragraph, and clearly inform the public of the metes and bounds of the claimed invention.

Regarding Claim 7, the Examiner contends that the phrase "inhibiting AIN" is unclear and questions whether "protein" or "mRNA or protein" is "inhibiting AIN." This rejection has been rendered moot by the cancellation of the rejected claim and the fact that the newly submitted claims do not recite the rejected language.

Regarding Claim 8, the Examiner contends that the claim is an incomplete method claim because the final step of the method does not produce the desired product. This rejection has been rendered moot by the cancellation of the rejected claim and the fact that newly submitted Claim 41 does not suffer from this alleged defect.

For the foregoing reasons, Applicant respectfully requests reconsideration and withdrawal of the claim rejections under 35 U.S.C. § 112, second paragraph.

Rejection under 35 U.S.C. § 112, ¶1: Enablement

Claims 1 and 3-8 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly not being enabled by the specification. The Examiner contends that the specification does not reasonably provide enablement for "unspecified conditions which inhibit Agrobacterium induced necrosis (AIN), or the broad scope of inhibiting agents or inhibitors." Office Action, p. 5, ¶ 6. The Examiner further contends that the "specification does not enable any person skilled in the art to which it pertains ... to make and use the invention commensurate in scope with these claims." Claims 1 and 3-8 are cancelled herein and have been replaced by Claims 20 and 26-41. The following remarks are therefore directed to Claims 20 and 26-41. Applicant respectfully traverses this rejection.

With due respect, Applicant objects to the Examiner's characterization of Applicant's invention for a plurality of reasons. In particular, the Examiner characterizes the "nature" of Applicant's invention by stating, *inter alia*,

> Applicant's invention is a method of transforming plants with Agrobacterium, whereby the transformation includes conditions which inhibit Agrobacterium induced necrosis (AIN) of the tissue. Only monocots appear to show this AIN, and AIN is thought to be one of the barriers to routine Agrobacterium-mediated transformation of monocots. Applicant proposes solving this problem by use of heat-shock to the tissue or by the use of chemical inhibitors or chemicals. The prior art teaches the use of AgNO3 as a chemical inhibitor to solve this problem. Applicant teaches heat-shock, AgNO3, and nucleotide sequences encoding p35, iap, or dad-1 to solve this problem. These three conditions of heat-shock, AgNO3, and the three nucleotide sequences, [sic] are very different and do not teach one of skill in the art how to predictably practice this invention commensurate in scope with these claims.

Office Action, p. 6.

Regarding the substance of Applicant's disclosure, Applicant submits that the teachings of the instant disclosure have at their core the surprising discovery that *Agrobacterium* induces programmed cell death or apoptotic necrosis in plant cells, a process that Applicant has termed *Agrobacterium*-induced necrosis (AIN). This finding stimulated the further discovery that the inhibition of AIN during *Agrobacterium*-mediated

plant transformation can, *inter alia*, enhance transformation effectiveness and/or efficiency. Given the knowledge that *Agrobacterium* induces a programmed cell death or apoptotic necrosis pathway in plant cells or tissues, any number of ways of inhibiting the pathway may be devised. That is, once Applicant accomplished the inventive aspect of discovering the source of the problem, a variety of solutions would become apparent to those of ordinary skill in the art, and, coupled with Applicant's disclosure, the implementation of such solutions would require no more than routine experimentation.

Applicant's disclosure describes various conditions for inhibiting AIN (*e.g.*, various chemical compounds, nucleotide sequences, and heat shock). See, *e.g.*, Specification, p. 3-6. Applicant provides extensive working examples (Specification, pp. 8-36), describing, *inter alia*, exposure to heat-shock or $AgNO_3$ and the delivery/expression of nucleotide sequences encoding *p35*, *iap*, or *dad-1*. Each of these examples is intended as merely that; it exemplifies how one skilled in the art may practice Applicant's invention. Applicant's claimed invention should not be so limited. While the exemplified conditions for inhibiting AIN may be viewed as being different from each other, they do, in fact, teach one of skill in the art how to predictably practice the invention commensurate in scope with the amended claims. Indeed, it is the very breadth of Applicant's representative number of species that more than justifies Applicant's claimed genus, that is, a condition of a plant cell or tissue that inhibits a programmed cell death or apoptotic necrosis mechanism capable of being induced by exposure of that plant cell or tissue to *Agrobacterium*.

Applicant respectfully submits that the Office has not met its burden of showing that the practice of the claimed invention would require undue experimentation. The Examiner contends that the "enablement issues" presented by the claimed invention are

> "conditions which inhibit," "agent," "a nucleotide sequence," and "ethylene inhibitors, ethylene synthesis inhibitors, gibberellin antagonists, and phosphatase inhibitors."

Office Action, p. 5, ¶ 6.

Contrary to the Examiner's allegations, Applicant's disclosure teaches far more than "two conditions for inhibition of AIN: heatshock treatment, and the use of silver nitrate as a

chemical ethylene inhibitor" (Office Action, p. 6, lines 18-19) and provides more than a reasonable amount of guidance and direction to enable the practice of the claimed invention without undue experimentation. Specifically, the disclosure provides a clear and exact description of certain molecular and biochemical features of AIN. For example, the specification teaches that AIN is characterized by

> DNA cleavage [of the plant genome] into oligonucleosomal fragments and defined morphological changes. In maize cells exposed to *Agrobacterium*, Ca^{2+} increases the intensity of DNA fragmentation whereas Zn^{2+} has the opposite effect, which parallels the effect of these divalent cations on endogenous endonucleases responsible for DNA cleavage during apoptosis in animal cells. This fragmentation is also decreased by addition of cycloheximide, which is evidence that the process requires *de novo* gene expression.

Specification, p. 2, lines 16-21. The specification also provides considerable guidance and direction regarding the use of various assays to detect and/or measure AIN or the inhibition thereof. In particular, detailed description and guidance are provided for the use of a DNA fragmentation assay (Specification, *e.g.*, pp. 10 and 19), a callus formation assay (Specification, *e.g.*, pp. 12-13 and 22-28), a callus proliferation assay (Specification, *e.g.*, pp. 12-13 and 22-28), and a GUS expression assay (Specification, *e.g.*, pp. 17-22).

Further, the specification teaches that AIN can be inhibited by exposing plant cells or tissues to *Agrobacterium* under a variety of conditions. One such condition is heat shocking the plant cells and tissues before co-cultivation with *Agrobacterium*. As described in the specification,

> AIN is reduced or inhibited by heat-shock treatment of the plant tissue to be transformed prior to co-cultivation with *Agrobacterium*. The heat shock treatment is performed at 40-50°C, preferably at 42-48°C, 45°C being a preferred temperature for maize. The treatment lasts for 2-10 minutes and preferably 4-8 minutes.

Specification, p. 4, ¶ 1.3.

Other disclosed conditions for inhibiting AIN include culturing the plant cells or tissues in the presence of various AIN-inhibiting chemicals. As demonstrated by the following passages from the specification, and contrary to the Examiner's allegations (see

Office Action, p.7, lines 12-15), Applicant teaches far more than simply the use of $AgNO_3$ as a chemical inhibitor. Applicant's disclosure provides various examples of ethylene inhibitors, ethylene synthesis inhibitors, gibberellin antagonists, and phosphatase inhibitors. As stated in the specification, the disclosure provides

> [a] method of transforming a plant cell with a gene of interest, comprising exposing said plant cell to *Agrobacterium* under conditions which inhibit AIN such as the presence of an AIN-inhibiting agent ....
>
> In one embodiment ..., the AIN-inhibiting agent is a chemical inhibitor. The chemical inhibitor is preferably a compound selected from the group consisting of ethylene inhibitors (e.g., 2,5- norbornadiene, norbornene, silver thiosulfate, and silver nitrate), ethylene synthesis inhibitors (e.g., aminoethoxyvinylglycine (AVG), cobalt salts, acetyl salicylic acid, or salicylic acid), gibberelin antagonists (e.g., abscisic acid (ABA)) and phophatase inhibitors (e.g., okadaic acid). Most preferably, the chemical inhibitor is an ethylene inhibitor, preferably silver nitrate. Proteins and peptides can act as chemical inhibitors as well. Examples are naturally occurring proteins such as DAD-1, the baculovirus inhibitors of apoptosis (IAPs), baculovirus p35, or synthetic peptide analogs of caspases capable of triggering apoptosis. A chemical inhibitor is suitably present in an effective concentration, e.g., for silver nitrate in a concentration of from 0.1 to 20 mg/l, preferably 1 to10 mg/l.

Specification, p. 3, ¶¶ 1 and 1.1.

The Examiner contends, "Applicants teach no examples of ethylene synthase inhibitors, gibberellin antagonists or phosphatase inhibitors." Office Action, p. 7. However, as demonstrated above, the specification does, in fact, teach examples of these chemical AIN inhibitors (*e.g.*, aminoethoxyvinylglycine, cobalt salts, acetyl salicylic acid, or salicylic acid; abscisic acid; and okadaic acid; respectively). Since the Examiner has not offered sufficient evidence to doubt Applicant's presumptively accurate disclosure, the specification must be taken as enabling. *In re Marzocchi*, 439 F.2d 220, 223 (C.C.P.A 1971).

Contrary to the Examiner's implied allegation that "Applicant teaches [only] the coding sequences of p35, iap, and dad-1" and that "[n]o guidance is given other than [these sequences]" (Office Action, from p. 7, line 21 to p. 8, line 1), the specification provides more than a reasonable amount of guidance regarding the use of a variety of nucleotide sequences.

The specification teaches a variety of exemplary coding sequences, such as, for example, bcl-1, p35, pIAP, nahG, dad-1, and mlo. As described in the specification,

> In an alternative embodiment of this method, the AIN-inhibiting agent is a nucleotide sequence. The AIN-inhibiting nucleotide sequence may inhibit AIN directly or by encoding an AIN-inhibiting mRNA coding for an AIN-inhibiting protein. For example, it may be an antisense oligonucleotide or a gene encoding antisense mRNA, which is antisense to a gene encoding a necrosis associated enzyme (e.g., protease, kinase, or phosphatase) or regulatory protein. Alternatively, it may comprise the coding region of a gene capable of inhibiting apoptosis under control of a promoter capable of expression in plants, e.g., a coding region of a mammalian bcl-1 gene under control of a promoter capable of expression in plants, a coding region of an apoptosis-inhibiting gene from a baculovirus such as p35 or pIAP, or a gene capable of suppressing disease response in plants, e.g., nahG, dad-1, or mlo. An AIN-inhibiting nucleotide sequence expressing an AIN-inhibiting protein may optionally be adapted for expression in the host plant by making a synthetic nucleotide sequence encoding the same protein but using codons which are preferred by the host plant and avoiding nucleotide sequences, e.g., polyadenylation signals or splice sites within the coding region, which may affect optimal expression in the host plant, e.g., analogously to the methods described in US 5,380,831 or US 5,610,042.

Specification, pp. 3-4, ¶ 1.21.

Further guidance provided by the specification for inhibiting AIN include delivering to, or expressing in, the plant cells or tissues various AIN-inhibiting nucleotide sequences under a variety of conditions. For example, AIN-inhibiting nucleotide sequences may be stably or transiently transformed into plant cells or tissues, and the nucleotide sequences may comprise antisense sequences. Specifically, the specification states:

> The AIN-inhibiting nucleotide sequence may be stably incorporated into the genome of the plant to be transformed or may be only transiently present and operable, e.g., at or around the time the cell is exposed to the *Agrobacterium*. Transient expression can be obtained, e.g., using an gemini viruses in tandem such that a viral replicon that carries the AIN-inhibiting nucleotide sequence may replicate in the cell. In this system, the virus typically will not integrate into the plant genome but will replicate to a high copy number and provide a high level of transient expression. Cells thus primed to be resistant to necrosis can then be transformed using

> *Agrobacterium* having Ti plasmids comprising the gene of interest, which will be incorporated into the genome, while the virus is diluted through regeneration and will not be transmitted to the seed. Thus the progeny and descendants of the infected plant are stably transformed with the gene of interest but not with the AIN-inhibiting nucleotide sequence. Transient expression may alternatively be obtained by introducing short AIN-inhibiting oligonucleotide sequences into the plant, e.g., antisense sequences.

Specification, p. 4, ¶ 1.22.

The Examiner contends, "Applicant does not give guidance for what is special about these conditions, which allow them to function in the desired manner." Office Action, p. 6. However, in view of Applicant's teachings regarding the nature of AIN, one skilled in the art would have appreciated that conditions known to affect or inhibit programmed cell death or apoptotic necrosis in other contexts could be used to counteract AIN. That is, at the time of filing of the instant application, it was known in the art that programmed cell death and apoptotic necrosis mechanisms occurred in a variety of cell types, including animal cells and plant cells, and that such mechanisms could be inhibited in a variety of ways. See, *e.g.*, Samali and Cotter (1996) *Exp. Cell Res.* 223(1):163-170 (describing how the induced production of heat shock proteins in mammalian cells increases resistance of those cells to apoptosis); Orzaez and Granell (1997) Plant J. 11(1): 137-144 (describing cells of the senescent pea carpel as having characteristics of apoptotic cells and the use of ethylene action inhibitors, such as silver thiosulphate and 2,5-norbornadiene, to inhibit senescence in these cells); and Wang et al. (1996) Plant Mol. Biol. 32:1125-1134 (describing the inhibitory effect of gibberellin antagonists, such as abscisic acid, and phosphatase inhibitors, such as okadaic acid, on apoptosis in barley cells). With the exception of the article by Samali and Cotter, which is enclosed herewith as Exhibit A, all of these references were submitted with the Information Disclosure Statement that was filed on April 4, 2001.

In addition to all of the above, the specification discloses numerous working examples in which several of the disclosed conditions are used to inhibit AIN in various plant tissues and cells. There are examples of using heat shock before inoculation with *Agrobacterium* to inhibit AIN in maize embryos and embryogenic calli (Specification, *e.g.*,

pp. 17-21), as well as in wheat embryos and embryogenic calli (Specification, pp. 21-22). There are examples of delivering and/or expressing AIN-inhibiting nucleotide sequences in maize embryos or embryogenic calli before, as well as concurrently with, *Agrobacterium* co-cultivation. The exemplified AIN-inhibiting nucleotide sequences include two apoptosis inhibiting genes, *iap* and *p35*, and a plant disease response gene, *dad*-1. Specification, pp. 22-28. There are also examples of using an AIN-inhibiting chemical, silver nitrate, either before or immediately after *Agrobacterium* co-cultivation, to inhibit AIN in maize immature embryos and embryogenic calli. Specification, *e.g.*, pp. 11-16.

Applicant respectfully submits that the working examples, in conjunction with the remainder of the disclosure, provide more than a reasonable amount of guidance and direction and that one skilled in the art would have been able to practice the claimed invention by employing no more than routine experimentation. Specifically, in view of the specification's detailed guidance and direction regarding the detection of AIN, one of ordinary skill in the art would have been able to determine whether AIN occurs in an *Agrobacterium*-mediated transformation reaction by employing no more than routine experimentation. Further, in view of the specification's considerable guidance and direction regarding the various disclosed conditions for inhibiting AIN, the skilled artisan would have been able to inhibit AIN in an *Agrobacterium*-mediated transformation reaction by employing no more than routine experimentation.

The Examiner contends,

> [i]t is unpredictable that any given condition would function as desired. Finding proper conditions would require a myriad of experiments of a multiplicity of conditions. In fact it would require an infinite number of experiments to find conditions which function in the desired manner. This would require excessive experimentation, and impose undue burden on one of ordinary skill in the art ...
>
> ... [Further, for] the highly specialized function of inhibiting AIN, it is unpredictable that any given compound would function in the desired manner. So one of ordinary skill would have to screen many chemicals at many different concentrations and conditions, to practice the claimed invention, imposing undue burden and excessive experimentation ...
>
> ... [In addition,] it is unpredictable that any given sequence would function in the desired manner. It would require many experiments of

> different types of nucleic acids, of many different lengths, of many different sequences. In fact it would require that [sic] an infinite number of experiments, to find nucleic acids which function in the desired manner. This would require excessive experimentation, and impose undue burden on one of ordinary skill in the art.

Office Action, pp. 7-8.

The Examiner is reminded that absolute predictability is not required to satisfy the enablement requirement of 35 U.S.C. § 112. The Examiner is also reminded that "[e]nablement is not precluded by the necessity for some experimentation such as routine screening." *In re Wands*, 858 F.2d 731, 736-737 (Fed. Cir. 1988). In fact, "a considerable amount of experimentation is permissible, if it is merely routine, or if the specification provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *Id.* at 737 (internal citation omitted). Thus, even where the practice of non-exemplified embodiments of the claimed invention might require some experimentation, Applicant respectfully submits that, in view of the specification's considerable teachings regarding the molecular and biochemical features of AIN and the conditions (including chemical compounds and nucleic acid sequences) for inhibiting AIN, carrying out any such experimentation would have been routine.

For the foregoing reasons, Applicant respectfully submits that the instant specification, coupled with what was known in the art at the time of filing, would have provided sufficient guidance to enable one of ordinary skill in the art to practice the claimed invention without undue experimentation. Accordingly, the claimed invention is enabled, and Applicant respectfully requests reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, first paragraph.

Rejection under 35 U.S.C. § 102: Goodman

Claims 1, 3, 4, and 6-8 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Goodman *et al.*, U.S. Patent. No. 4,956,282, issued September 11, 1990 ("Goodman"). The Examiner states,

> Goodman teaches a method of transforming a plant cell or tissue with a gene of interest, comprising exposing said plant cell or tissues to

> Agrobacterium, wherein said Agrobacterium comprises a vector comprising said gene of interest (col. 8, lines 40-50). Goodman further teaches chemicals and nucleotide sequences. Since the conditions are unspecified, and the chemicals and nucleotide sequences are unspecified, and there is no evidence of Agrobacterium induced necrosis, the conditions utilized by Goodman, [sic] must inherently inhibit AIN.

Office Action, p. 9, ¶ 8. Claims 1, 3, 4, and 6-8 are cancelled herein and have been replaced with claims 20, 26, and 32-41. The following remarks are therefore directed to Claims 20, 26, and 32-41. Applicant respectfully traverses this rejection.

Applicant's claimed invention is directed to methods for transforming a plant cell or tissue susceptible to *Agrobacterium*-induced necrosis with a nucleotide sequence of interest and producing a fertile transgenic plant therefrom, comprising, *inter alia*, "manipulating a condition of a plant cell or tissue to inhibit a programmed cell death or apoptotic necrosis mechanism capable of being induced by exposure of said plant cell or tissue to *Agrobacterium*."

Goodman discloses methods for transforming plant cells with nucleotide sequences that encode physiologically active mammalian proteins. Specifically, Goodman teaches the *Agrobacterium*-mediated transformation of tobacco cells (col. 8, lines 40-50) with a murine γ-interferon gene (see, *e.g.*, col. 7, line 31).

Applicant submits that the Examiner has not established that a programmed cell death or apoptotic necrosis was capable of being induced by exposure of the tobacco cells or tissue discussed in Goodman to *Agrobacterium*. It is axiomatic that inherency may not be established by mere probabilities or possibilities. That is to say, the mere fact that a certain thing may result from a given set of circumstances is <u>not</u> sufficient to establish inherency. Indeed, Applicant respectfully submits that it appear that the Examiner's own statement set forth at page 8 of the Office Action would contradict the position now taken. At page 8 of the Action, the Examiner states that "[t]he conditions of Agrobacterium induced necrosis are only exhibited in monocot plants." If one assumed, *arguendo*, that this statement is true, then that would not support the apparent basis of the Examiner's rejection of the claimed invention under § 102(b), *i.e.*, inherency due to Goodman, since Goodman discloses transformation of a dicot.

Accordingly, the Examiner has not established that the claimed invention is anticipated by Goodman. Applicant therefore respectfully requests reconsideration and withdrawal of this rejection under 35 U.S.C. § 102(b).

Rejection under 35 U.S.C. § 102: De Block

Claims 1 and 3-8 stand rejected under 35 U.S.C. § 102(b) as being anticipated by De Block *et al.*, Plant Physiol. 91:694-701 (1989) ("De Block"). The Examiner contends,

> De Block teaches a method of transforming plant tissue, and a method of making a fertile, transgenic plant (Figure 1, p. 698, final paragraph p. 698), by exposing the tissue to Agrobacterium transforming a plant cell or tissue with a gene of interest comprising exposing said plant cell wherein said Agrobacterium comprises a vector comprising said gene of interest (Abstract, p. 694). De Block also teaches the ethylene inhibitor AgNO3 (Abstract; p. 695, 1st paragraph and Table II). Since there is no evidence of Agrobacterium induced necrosis, the conditions utilized by De Block, [sic] must inherently inhibit the AIN.

Office Action, p. 9, ¶ 9. Claims 1 and 3-8 are cancelled herein and have been replaced with claims 20 and 26-41. The following remarks are therefore directed to Claims 20 and 26-41. Applicant respectfully traverses this rejection.

As stated above, Applicant's claimed invention is directed to methods for transforming a plant cell or tissue susceptible to *Agrobacterium*-induced necrosis with a nucleotide sequence of interest and producing a fertile transgenic plant therefrom, comprising, *inter alia*, "manipulating a condition of a plant cell or tissue to inhibit a programmed cell death or apoptotic necrosis mechanism capable of being induced by exposure of said plant cell or tissue to *Agrobacterium*."

Applicant submits that the Examiner has not established that a programmed cell death or apoptotic necrosis was capable of being induced by exposure of the *Brassica* cells or tissue discussed in De Block to *Agrobacterium*. It is axiomatic that inherency may not be established by mere probabilities or possibilities. That is to say, the mere fact that a certain thing may result from a given set of circumstances is not sufficient to establish inherency. Indeed, Applicant respectfully submits that it appear that the Examiner's own statement set forth at page 8 of the Office Action would contradict the position now taken.

At page 8 of the Action, the Examiner states that "[t]he conditions of Agrobacterium induced necrosis are only exhibited in monocot plants." If one assumed, *arguendo*, that this statement is true, then that would not support the apparent basis of the Examiner's rejection of the claimed invention under § 102(b), *i.e.*, inherency due to De Block, since De Block discloses transformation of a dicot.

Accordingly, the Examiner has not established that the claimed invention is anticipated by De Block. Applicant therefore respectfully requests reconsideration and withdrawal of this rejection under 35 U.S.C. § 102(b).

Rejection for Obviousness-Type Double Patenting

Claims 1-8 stand rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over Claims 1-48 of U.S. Patent No. 6,162,965. The Examiner states that although the conflicting claims are not identical, they are not patentably distinct from each other, because the claims of the instant application are broader and, therefore, encompass the '965 claims. The Examiner contends that the species claims of the '965 Patent render obvious the genus claims of the instant application.

Applicant respectfully requests that this rejection be held in abeyance until allowable subject matter has been indicated by the Examiner.

CONCLUSION

Pursuant to the foregoing remarks, Applicant respectfully submits that all of the pending claims fully comply with 35 U.S.C. § 112 and are allowable over the prior art of record. No new matter is added by this amendment. Reconsideration of the application and allowance of all pending claims is earnestly solicited. Should the Examiner wish to discuss any of the above in greater detail or deem that further amendments should be made to improve the form of the claims, then the Examiner is invited to telephone the undersigned at the Examiner's convenience.

Attached hereto is a marked-up version of the changes made to the specification and the claims by the current amendment. The attached marked-up pages are captioned

"**Version With Markings To Show Changes Made**". Entry of the amendments is respectfully requested.

Respectfully submitted,

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Date: December 20, 2002

Version With Markings To Show Changes Made

In the Specification

The specification has been amended as follows:

On page 1, the title has been amended as follows:

Methods for Inhibiting *Agrobacterium*-Induced Necrosis During Plant Transformation [Methods]

The first paragraph on page 1 has been amended as follows:

This application [claims the benefit of U.S. serial number 08/867,869, filed June 2, 1997 as a regular application and subsequently converted to a provisional application] is a continuation of U.S. Application Serial No. 09/089,111, now U.S. Patent No. 6,162,965, filed June 2, 1998, which claims the benefit of U.S. Provisional Application Serial No. 60/098,564, filed June 2, 1997, both of which are herein incorporated by reference in their entireties.

On page 3, the second paragraph has been amended as follows:

1.1. In one embodiment of the foregoing method, the AIN-inhibiting agent is a chemical inhibitor. The chemical inhibitor is preferably a compound selected from the group consisting of ethylene inhibitors (e.g., 2,5- norbornadiene, norbornene, silver thiosulfate, and silver nitrate), ethylene synthesis inhibitors (e.g., aminoethoxyvinylglycine (AVG), cobalt salts, acetyl salicylic acid, or salicylic acid), [gibberelin] gibberellin antagonists (e.g., abscisic acid (ABA)) and [phophatase] phosphatase inhibitors (e.g., okadaic acid). Most preferably, the chemical inhibitor is an ethylene inhibitor, preferably silver nitrate. Proteins and peptides can act as chemical inhibitors as well. Examples are naturally occurring proteins such as DAD-1, the baculovirus inhibitors of apoptosis (IAPs), baculovirus p35, or synthetic peptide analogs of caspases capable of triggering apoptosis. A chemical inhibitor is suitably present in an

effective [concentration ,] concentration, e.g., for silver nitrate in a concentration of from 0.1 to 20 mg/l, preferably 1 to10 mg/l.

The paragraph bridging pages 3 and 4 has been amended as follows:

1.2.1. In an alternative embodiment of this method, the AIN-inhibiting agent is a nucleotide sequence. The AIN-inhibiting nucleotide sequence may inhibit AIN directly or by encoding an AIN-inhibiting mRNA coding for an AIN-inhibiting protein. For example, it may be an antisense oligonucleotide or a gene encoding antisense mRNA, which is antisense to a gene encoding a necrosis associated enzyme (e.g., protease, kinase, or phosphatase) or regulatory protein. Alternatively, it may comprise the coding region of a gene capable of inhibiting apoptosis under control of a promoter capable of expression in plants, e.g., a coding region of a mammalian bcl-1 gene under control of a promoter capable of expression in plants, a coding region of an apoptosis-inhibiting gene from a baculovirus such as p35 or pIAP, or a gene capable of suppressing disease response in plants, e.g., nahG, dad-1, or mlo. An AIN-inhibiting nucleotide sequence expressing an AIN-inhibiting protein may optionally be adapted for expression in the host plant by making a synthetic nucleotide sequence encoding the same protein but using codons which are preferred by the host plant and avoiding nucleotide sequences, e.g., polyadenylation signals or splice sites within the coding region, which may affect optimal expression in the host plant, e.g., analogously to the methods described in [US] U.S. Patent No. 5,380,831 or [US] U.S. Patent No. 5,610,042.

On page 7, the first full paragraph has been amended as follows:

The *Agrobacterium* is preferably selected from *A. tumefaciens* and *A. rhizogenes*. Preferably the *Agrobacterium* strain is an *A. tumefaciens* strain, most preferably a nopaline-utilizing strain. When the *Agrobacterium* strain is an *A. rhizogenes* strain, it is preferably an

agropine- or mannopine-utilizing strain. Most preferably, the *Agrobacterium* is an *Agrobacterium* which does not induce necrosis in Gramineae, e.g., an *Agrobacterium* selected from *A. tumefaciens* strains A and B. *Agrobacterium* strains A and B have been deposited with the American Type Culture Collection (ATCC) [12301 Parklawn Drive, Rockville, Maryland 20852] 10801 University Boulevard, Manassas, Virginia 20110 / USA, under ATCC Designation numbers 55964 and 55965 respectively on May 2, 1997, pursuant to the Budapest Treaty.

In the Claims

Claims 1-19 have been cancelled without prejudice or disclaimer.

Claims 20-41 have been added.

EXHIBIT A

EXPERIMENTAL CELL RESEARCH 223, 163–170 (1996)
ARTICLE NO. 0070

Heat Shock Proteins Increase Resistance to Apoptosis

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Heat shock treatment of cells increases their survival and resistance to apoptosis. The kinetics of development of this resistance correlates with the kinetics of synthesis of heat shock proteins (hsps). U937 and Wehi-s cells were cultured for 1 h at 42°C, conditions which induced the synthesis of heat shock proteins 27, 70, and 90. The cells were subsequently permitted to recover for a 2-h period, prior to exposure to the apoptosis inducing agents actinomycin-D (5 μg/ml), camptothecin (5 μg/ml), and etoposide (25 μg/ml). Apoptosis was determined by both DNA fragmentation and flow cytometric analysis. Heat-shocked cultures had a smaller number of apoptotic cells compared to control cultures when both were exposed to apoptosis inducing stimuli. Transfected Wehi-s cells constitutively overexpressing human hsp 70 or 27 were then examined for their resistance to apoptosis induced by these drugs. Using the MTT assay, hsp 27 and 70 overexpressing cells exhibited an increased resistance to cell death when compared to the parental line. The parental line demonstrated features of apoptosis, that is, cell shrinkage and single- and double-strand DNA breaks. Taken together these results demonstrate that an increase in cellular levels of hsp 27 or 70, either by a mild heat shock treatment or by stable transfection, increases the resistance of U937 and Wehi-s cells to apoptotic cell death.

INTRODUCTION

Apoptosis and necrosis are two biochemically and morphologically distinct processes by which cells die. Cells under normal conditions and cell lines in culture undergo apoptosis when exposed to a variety of cytotoxic agents [3–5]. Apoptosis occurs mainly under physiological conditions and allows the cell to actively participate in its own death. Morphologically this process is characterized by cell shrinkage, chromatin condensation [6, 7], and DNA fragmentation of varying degrees, i.e., single-strand breaks, double-strand and high-molecular-weight fragmentation. These sequence of events may require macromolecular synthesis [7, 8], though this requirement is not absolute in many circumstances [9]. In necrosis, however, the cell membrane is rapidly damaged, ATP production drops, mitochondria swell, and finally the cell ruptures, releasing pro-inflammatory agents.

Cells in culture appear to respond to changes in their normal growth environment in a graded fashion. At early stages of low levels of stress the "stress response" is initiated [10, 11]. This involves the synthesis of a set of evolutionary conserved proteins, known as stress-induced or heat shock proteins [12, 13]. The main function of these proteins in time of stress is to afford protection to cells. When the stress element is removed these cells continue to function normally and the hsp[2] levels drop back to the normal levels. However, if the level of stress is increased, the presence of heat shock proteins may be unable to protect the cell, hsp synthesis stops, and at this stage the program for apoptosis is activated. As the stress level is further increased uncontrolled necrotic death becomes the prominent mode of cell death [5].

There is strong evidence suggesting that induction of hsp's coincides with acquisition of tolerance to higher doses of the stress which otherwise may be lethal to the cell [14]. Heat-shocked or thermotolerant cells show a greater degree of resistance to environmental stress [13, 15] and to cell death in general. Apoptosis induced by hyperthermia [1] or growth factor withdrawal [2] appears to be inhibited in heat-shocked cells, suggesting a possible role for heat-shock-induced stress proteins in the resistance mechanism. There is also evidence suggesting that heat shock proteins are involved in drug resistance [16] and that heat-shocked/thermotolerant [17–19] or hsp transfected cells [20] become more resistant to cytotoxic effects of some anticancer drugs, which otherwise are capable of inducing apoptosis [4, 5, 21].

Many groups have carried out studies on thermotolerance and cell survival. However, very few have de-

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[2] The abbreviations used are: Act-D, actinomycin-D; BSA, bovine serum albumin; Camp, camptothecin; DMSO, dimethylsulfoxide; Etop, etoposide; hsp, heat shock protein.

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fined cell survival as resistance to apoptosis. In this paper we demonstrate that (a) hsp 27, 70, and 90 synthesis increases after a mild heat shock treatment and that (b) heat shock treatment renders cells more resistant to drug-induced apoptosis. (c) Finally, using hsp transfected cell lines we have further demonstrated that hsps 27 and 70 are both involved in resistance to apoptosis.

MATERIALS AND METHODS

Cell lines and culture conditions. The human monoblastoid cell line, U937 [22], the Wehi-s cells, a highly TNF-α sensitive subclone of the murine fibrosarcoma cell line Wehi-164, and transfected subclones, Wn-10X, Wn-113, and Wn-139 [15], were used throughout this study. The control Wn-10X clone was transfected with pSV2neo; Wn-113 overexpresses the human *hsp* 70 gene constitutively, whereas clone Wn-139 was stably transfected with human *hsp* 27 gene. All cells were grown in RPMI-1640 medium (Gibco Ltd., Paisley Scotland, UK), supplemented with 10% (v/v) fetal calf serum, (Biochrom KG, Germany), 1% (v/v) penicillin/streptomycin solution (Gibco). In order to maintain the neomycin-resistant transfected clones 200 μg/ml G418 (Sigma) was also supplemented to the medium for transfected cells. Cultures were maintained in a humidified incubator at 37°C in a controlled 5% CO_2/95% air atmosphere and were used for experiments during the exponential phase of growth.

Cells at a concentration of 1×10^6 cells/ml were heat shocked at the indicated temperatures in polystyrene culture flasks (Nunc); after the flasks were sealed with parafilm, they were fully immersed in a thermostated water bath.

Cell viability and morphology. Cell density was determined using an improved Neubauer hemocytometer and viability was assessed by their ability to exclude the vital dye trypan blue.

For morphological evaluation of the cultured cells, cytospins were prepared and stained with RAPI-DIFF II (Diagnostic Development, Southport, UK). The slide preparations were mounted using DPX mountant (BDH Chemicals, Dorset, UK) and percentage apoptosis was determined as previously described [21].

Western blot analysis of proteins. For Western blot analysis, the cells were lysed in Laemmli sample buffer [23] boiled for 10 min and sonicated. Lysates from 5×10^5 cells/lane were electrophoresed through 10% SDS–PAGE [23], the molecular weight markers were obtained from Amersham, United Kingdom. Proteins were transferred onto a nitro-cellulose filter using a transblotter overnight at 30 V. The filters were preblocked in blocking solution (5% w/v low-fat milk in PBS) for 1 h. hsp levels were detected by probing with either a 1:1000 dilution of the monoclonal anti-hsp 27 (clone G3.1) or anti-hsp 72 (clone C92F3A-5) or 1:5000 dilution of monoclonal anti-hsp 90 (clone 9D2) (StressGen Biotechnologies Corp., Canada). Monoclonal anti-β-actin (Sigma Chemicals Co., St. Louis, MO) (clone AC-15) at a dilution of 1:1000 was used as a control for protein loading.

Following 3 × 15 min washes (PBS, 0.2% Tween), filters were incubated with the blocking solution containing 1:1000 dilution of either the goat anti-mouse IgG or goat anti-rat IgG peroxidase conjugate (both from Sigma). After 3 × 15 min washes (PBS, 0.2% Tween) the proteins were detected using ECL-Western blot detection kit (Amersham, UK) and photographic plates (Kodak).

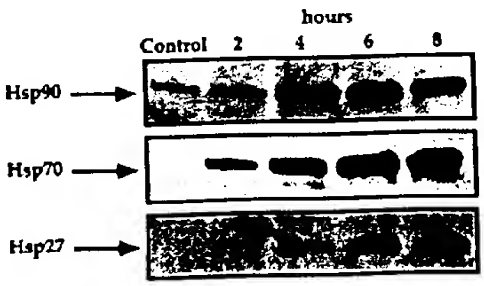

FIG. 1. Western blot analysis of hsp's 90, 70, and 27 in heat-shocked U937 cells. Cells were heat shocked for 1 h at 42°C, followed by incubation at 37°C for 2, 4, 6, and 8 h. Control lane is the proteins extracted from untreated cells.

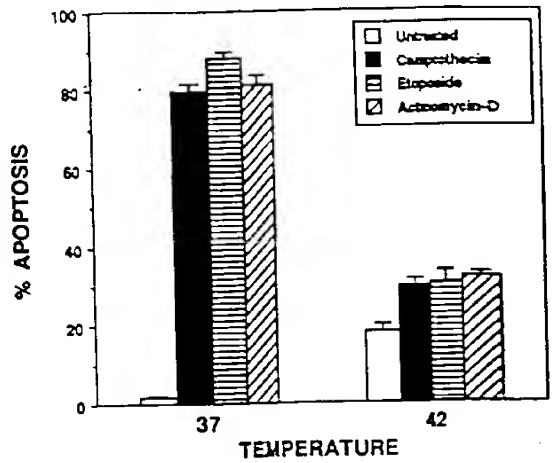

FIG. 2. Induction of apoptosis in normal and heat-shocked (for 1 h at 42°C and 2 h recovery at 37°C) U937 cells, treated with Act-D (5 μg/ml), Camp (5 μg/ml), or Etop (25 μg/ml) for 4 h. The percentage apoptosis was assessed by morphological examination of stained cytospin preparations on slides from three independent experiments and the results are expressed as the mean percentage ± SEM.

Induction of cell death. Cells were induced to undergo apoptosis in cultures by adding actinomycin-D (5 μg/ml), camptothecin (5 μg/ml), or etoposide (25 μg/ml) for the indicated time period. Actinomycin-D, camptothecin, and etoposide were supplied by Sigma prepared as stock solutions in DMSO and stored at −20°C.

MTT assay. The assay was carried out as described previously [24]. Briefly, cells were seeded in flat-bottomed 96-well plates (Nunc), at 1×10^4 cells/well 24 h prior to treatment. The cells were treated for various lengths of time with the agents indicated. Then 25 μl of 5 mg/ml MTT in dH_2O was added to each well. After 2 h at 37°C, 100 μl of lysing buffer was added. The buffer consisted of 20% w/v of SDS dissolved in 50% of each DMF and deionized water at 37°C; the pH was adjusted to 4.7 using acetic acid and HCl. The plates were incubated for a further 24 h before analysis on an ELISA reader at 570 nm.

DNA extraction and electrophoresis. DNA was isolated as described previously [25]. Briefly, 4×10^5 to 5×10^5 cells were harvested and incubated in 20 μl lysis buffer (20 m*M* EDTA, 100 m*M* Tris, 0.8% sodium lauryl sarcosinate) containing 10 μl of 1 mg/ml RNase A (prepared in 0.1 *M* sodium acetate, 0.3 m*M* EDTA, pH 4.8) at 37°C for 1 h. Subsequently a 10-μl aliquot of 20 mg/ml Proteinase K (made up in distilled water) was added to each sample and further incubated at 50°C for 2 h. Electrophoresis was carried out on 1.5% agarose gels in TBE (2 m*M* EDTA, pH 8.0, 89 m*M* Tris, 89 m*M* boric acid), a 3-μl aliquot of 10 mg/ml EtBr solution was added to 100 ml

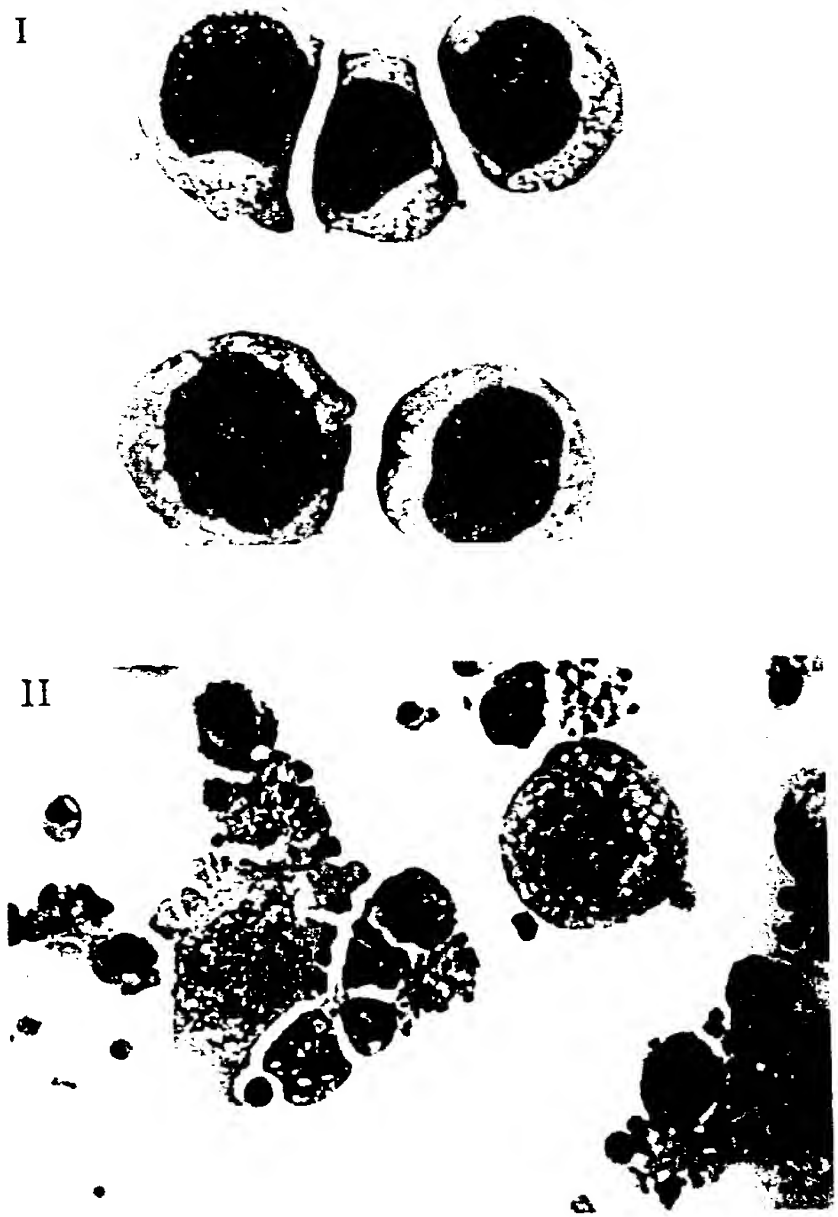

FIG. 3a. (I) Morphological features of U937 cells grown under normal culture conditions and (II) cells treated for 4 h with 5 μg/ml of Camp. Apoptotic cells and apoptotic bodies are clearly distinguishable in the photograph.

of 1.5% agarose prior to gel casting. Prior to loading the samples into dry wells 5 μl loading buffer (10 m*M* EDTA, 0.25% (w/v) bromophenol blue and 50% (v/v) glycerol) was added to each sample. Gels were cast in an apparatus supplied by CBS Scientific Co. California, and electrophoresis was conducted at 55V for 4 h. DNA was visualized by transillumination with UV light (302 nm).

DNA nick-end labeling. For *in situ* terminal deoxynucleotidyl transferase-mediated labeling of DNA nicking, we used a modified version of the assay previously described by Gorczyca *et al.* [26]. Cells (5×10^5/ml) were centrifuged at 1000 rpm for 5 min and resuspended in 1% paraformaldehyde in PBS (pH 7.4) and left for 15 min on ice. Fixed cells were then washed with PBS, before resuspension in 70% ice-cold ethanol. Cells were then stored overnight at −20°C. After a further washing they were resuspended in 50 μl reaction mixture containing 0.1 m*M* dithiothreitol, 0.05 mg/ml bovine serum albumin, 2.5 m*M* $CoCl_2$, 0.4 m*M* bio-16-dUTP and 0.1 U/ml TdT enzyme in 0.1 *M* Na cacodylate (pH 7.0) buffer. This mixture was incubated at 37°C for 30 min. Cells were then washed in PBS and resuspended in 100 μl staining buffer containing 2.5 mg/ml fluoresceinated avidin, 4× concentrated saline–sodium citrate buffer, 0.1% Triton X-100

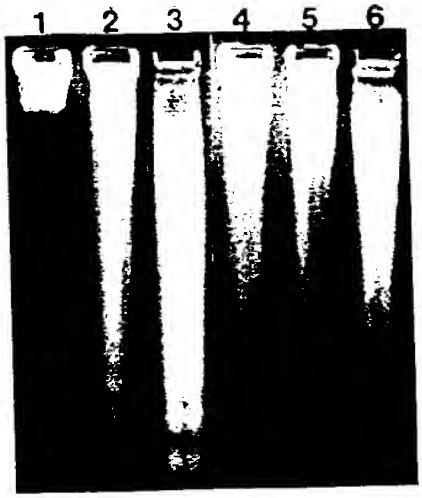

FIG. 3b. Photograph of a 1.5% agarose gel electrophoresis of DNA extracted from normal or heat-shocked U937 cells treated with topoisomerase inhibitors Camp or Etop. Lane 1, untreated U937 cells; lane 2, cells treated with Camp (5 μg/ml); lane 3, cell treated with 25 μg/ml of Etop; lane 4, cells heat shocked for 1 h at 42°C and allowed to recover for 6 h; lane 5, heat-shocked cells treated with 5 μg/ml of Camp for 4 h after 2 h recovery at 37°C; lane 6, heat-shocked cells treated with 25 μg/ml Etop. The ladder pattern is a result of ~200-bp internuclosomal DNA fragmentation, the biochemical hallmark of apoptosis.

and 5% (w/v) low-fat dry milk. Cells were incubated for 30 min at room temperature in the dark. Stained cells were washed in PBS before analysis for fluorescence, using a Becton Dickinson FACScan. Bio-16-dUTP, and the TdT-enzyme were obtained from Boehringer Mannheim. All other chemicals were obtained from Sigma.

Flow cytometry. The criteria for cell death as measured by flow cytometry was based on changes in light scattering properties of dead cells due to cell shrinkage and increased granularity [27]. Flow cytometric analysis was carried out using a FACScan flow cytometer equipped with Lysis II software (Becton Dickinson). For flow cytometry, cells were harvested at 1×10^6/ml, washed in PBS, and fixed in ice cold 70% ethanol for 30 min on ice.

RESULTS

Effect of Heat Shock on Cells in Culture

In our initial experiments, U937 cells were heat shocked for 1 h at 42°C and allowed to recover at 37°C for 2–8 h. This treatment induced synthesis of heat shock proteins, measured by an increase in cellular levels of hsp 27, 70, and 90 on Western blots (Fig. 1). A low background level of hsp 90 was detectable in nonstressed cells and this is in line with previous observations by others [11]. The cellular levels of the proteins reached a maximum after 6–8 h of recovery at normal culture conditions at 37°C. After 10 h there was a steady decline until the hsp levels returned to normal at 20–24 h (data not shown).

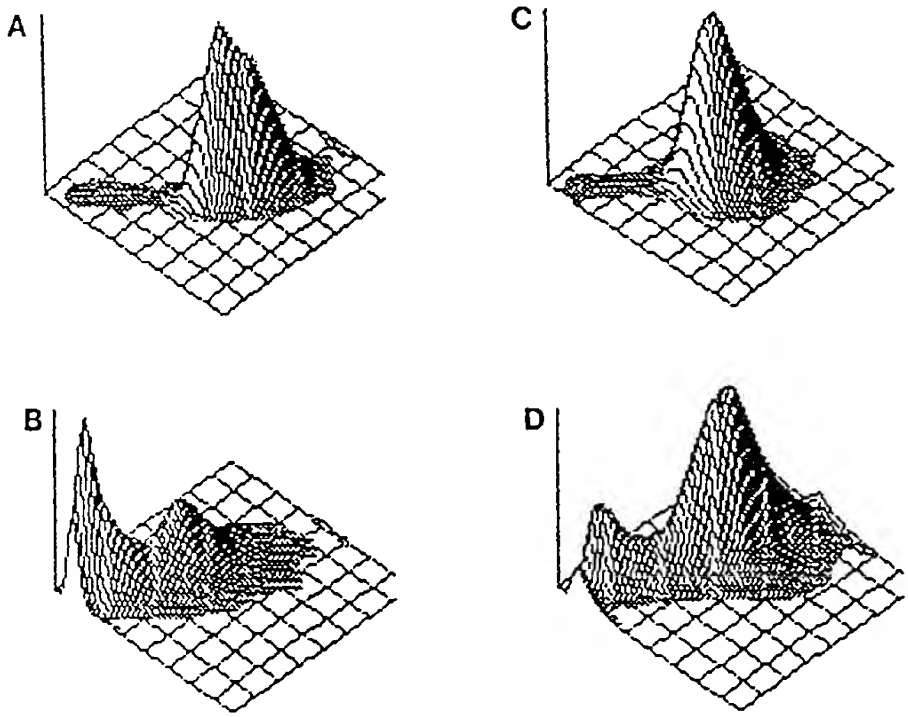

FIG. 4. Effect of Camp on light-scattering properties of U937 cells; (a) untreated control; (b) cells treated with 5 μg/ml Camp; (c) cells heat shocked for 1 h at 42°C and incubated at 37°C for 6 h; and (d) heat-shocked cells treated for 4 h with 5 μg/ml Camp. Cells were fixed in 70% ethanol and analyzed on a FACScan as described under Material and Methods.

To determine if this temporary elevation of hsp's induced a resistance to apoptosis, U937 cells heat shocked for 1 h with subsequent recovery period (2 h) were exposed to actinomycin-D, camptothecin, or etoposide for a further 4 h. The levels of apoptosis were measured by morphological analysis. Figure 2 demonstrates that this heat shock treatment induced a clear-cut resistance to apoptosis induced by all three stimuli.

U937 cells rapidly undergo apoptosis upon treatment with Camp. Figure 3a demonstrates classical morphological changes associated with apoptosis in U937. Internuclosomal DNA fragmentation was also detectable in these cells (Fig. 3b, lane 2). However, heat-shocked cells clearly demonstrate a lesser degree of DNA fragmentation after treatment with the drugs (Fig. 3b). The result from this experiment suggests that hsp's inhibit DNA fragmentation associated with cell death via apoptosis. Another characteristic feature of apoptotic cell death is the dramatic change in the cell volume [27]. Expression of hsp's also inhibits cell shrinkage in heat-shocked cells (Fig. 4). These experiments demonstrate that when the expression of hsp's are induced by a brief temperature insult, they protect the cell from apoptosis and prevent a number of key cellular changes associated with the process. Similar results were obtained with Wehi-s cells (data not shown).

Induction of Apoptosis in hsp Transfected Cells

Exposure of cells to a 1-h heat shock results in an elevation of hsp 27, 70, and 90 which correlates with a resistance to apoptosis. However, to unequivocally demonstrate that the observed protection seen was due to these proteins and not some other heat-shock-related event in the cell, we relied on hsp transfected cells. Wehi-s cells transfected with human *hsp* 27 (Wn-139) or *hsp* 70 (Wn-113) genes [15] and which therefore constitutively overexpress hsp 27 and hsp 70 were used in these experiments.

Using an MTT assay to detect cell death, we demonstrated that the clones overexpressing hsp 27 or 70

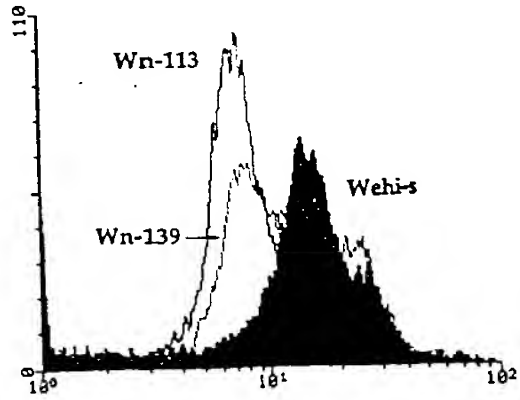

FIG. 6a. *In situ* terminal deoxynucleotidyl transferase assay of nick-end labeling of DNA. After treatment with 5 μg/ml of camptothecin for 8 h, 5×10^5 cells per sample were fixed and analyzed for DNA single-strand breaks as described under Material and Methods. Fluorescence was measured using Lysis II software on a FACScan. Diagram shows Wehi-s (parental cells), Wn-113 (hsp 70 transfected line), and Wn-139 (hsp 27 transfected line). For clarity, the profiles of Wn-10X (the control transfectants), which was identical to that of the Wehi-s cells, and the untreated cells are not shown.

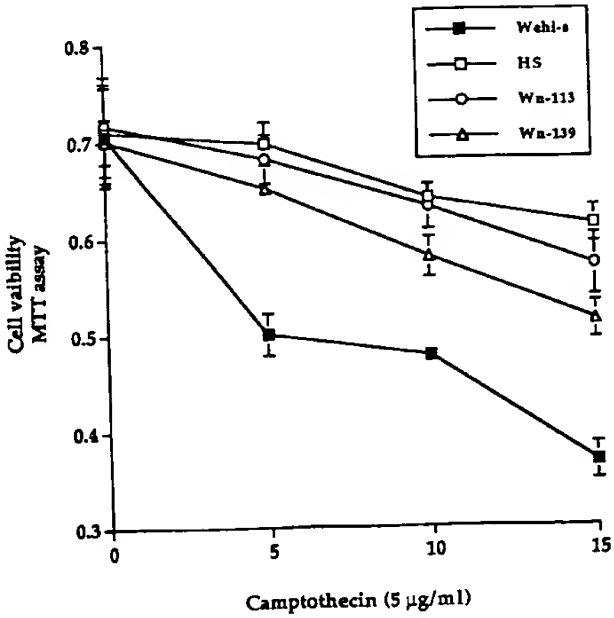

FIG. 5. Cell viability was determined after indicated time intervals by tetrazolium staining (MTT) and measuring the optical density at 570 nm. Wehi-s, heat-shocked Wehi-s (HS), Wn-113, and Wn-139 cells were treated with 5 μg/ml of Camp. The results are representative of three independent experiments ± SEM.

FIG. 6b. Agarose gel electrophoresis of extracted DNA from cells treated with 5 μg/ml Camp. Lane 1, Wehi-s cells; lane 2, Wn-113 cells; and lane 3, Wn-139 cells. The internucleosomal DNA fragmentation is detectable in lane 1 only.

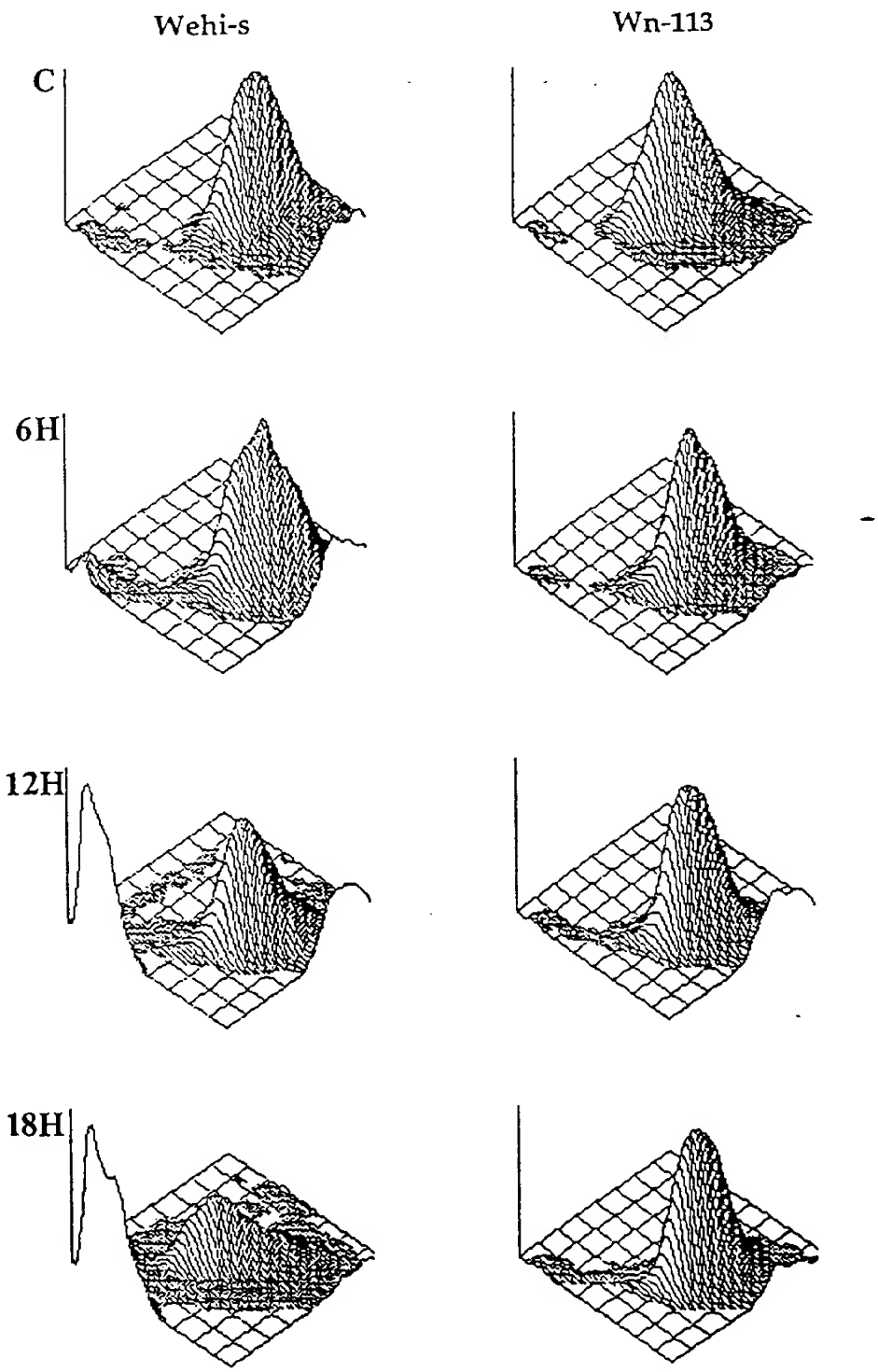

FIG. 7. Camp-induced changes in side-scatter profile of (a) Wehi-s cells and (b) hsp 70 transfected Wn-113 clone. Untreated control (C) and cells incubated with 5 μg/ml of Camp at 37°C for 6 (6H), 12 (12H), and 18 (18H) h were then fixed in 70% ice-cold ethanol before analysis on a FACScan equipped with Lysis II software.

exhibited a resistance to apoptosis very similar to that seen when untransfected cells were heat shocked (Fig. 5). The control clone Wn-10X transfected with pSV2neo alone showed no such resistance. These results were of extreme significance since they demonstrate that either hsp 27 or hsp 70 alone are able to inhibit cell death. The MTT assay is a convenient screening assay for the measurement of cell death but it does not discriminate between necrosis and apoptosis. To determine whether the expression of both of these hsp's could inhibit cell shrinkage and DNA fragmentation, associated hallmarks of apoptosis, the following experiments were carried out. The parental and the transfected clones were exposed to 5 μg/ml Camp and checked for single-strand DNA breaks, after an 8 h incubation period with the drug, using a flow cytometry *in situ* 3' nick-end labeling method. Figure 6a demonstrates a greater proportion of DNA strand breaks in the parental cells (Wehi-s) in comparison to the Wn-139 and Wn-113 clones. However, only the parental cells expressed double-stranded DNA fragmentation 24 h post-camptothecin treatment; in comparison Wn-113 and Wn-139 cells did not express any detectable internucleosomal DNA fragmentation using agarose gels (Fig. 6b). The Wn-113 clone also exhibited resistance to cell shrinkage whereas the parental line expressed the typical shrinkage pattern associated with apoptosis, after treatment with Camp (Fig. 7). Similar results were obtained with Wn-139 clone (data not shown).

DISCUSSION

A number of previous studies have demonstrated that a mild heat shock induces the rapid synthesis of a number of stress proteins including hsp's 27, 70, and 90 in cells [1, 2, 15]. It has also been demonstrated that cells in which heat shock proteins have been induced are somewhat resistant to cell death induced by hyperthermia [1] or growth factor withdrawal [2]. In the present study we clearly demonstrate that cells with an elevated expression of heat shock proteins, induced by either mild hyperthermia or by transfection of heat shock protein genes, *hsp* 27 or *hsp* 70, are resistant to apoptosis induced by a variety of toxic agents.

Our initial experiments demonstrated that when U937 and Wehi-s cells were heat shocked by culturing at 42°C for 1 h expression of the three hsp's increased, reaching a maximum after 8 h, following a return of the cells to 37°C (Fig. 1). Following this there was a slow decline until they reached normal levels again at 24 h. When these heat-shocked cells were exposed to either Act-D or the topoisomerase inhibitors Camp and Etop they showed a resistance to cell death via apoptosis when compared to cells which were not heat-shocked. This suggested that hsp's were able to protect the cells from apoptosis. This is in agreement with previous studies [1, 2]. However, it was possible that other heat-shock-associated changes in the cells, apart from the increased synthesis of hsp's was affecting the resistance seen. In addition, it was not possible using this system to dissect the contributions made by each of the individual hsp's. To overcome this limitation we used Wehi-s cells which had been transfected with *hsp* 27 and 70 genes. This allowed us to examine the contribution of hsp 27 and 70 to the observed induction of resistance to apoptosis.

Wehi-s cells transfected with hsp 27 or hsp 70 proved to be the ideal system for our studies. The transfected cells were subjected to the induction of apoptosis using the same experimental conditions described for the data expressed in Figs. 2, 3 and 4. In both cases we saw resistance to apoptosis indicating a role for both hsps. The mechanisms by which either of these genes induces a state of resistance to apoptosis is unclear, but may involve modulating regulatory genes such as abl, bcl-2, myc, or p53.

Mailhos *et al.* [2] demonstrated that transfection of neuronal cells with hsp 70 and hsp 90 protected them against thermal stress, but not apoptosis induced by other agents [28]. These results appear to be in contradiction with their earlier findings that heat shock inhibits apoptosis [2]. They have suggested that since heat shock inhibits synthesis of most cellular proteins, but enhances hsp synthesis [11, 13], it is possible that heat shock may protect against apoptosis, by inhibiting the synthesis or modification of one or more of the death proteins.

The fact that hsps inhibit apoptosis induced by a variety of cytotoxic agents may also indicate existence of a common death mechanism. The potential role of hsp's 70 and 27 in resistance to apoptosis and their interactions with genes regulating apoptosis in a number of different cell systems is currently under investigation in our laboratory.

We are grateful to Dr. M. Jaattela, Danish Cancer Society, in Copenhagen, for supplying the Wehi-s and transfected clones. This work was supported by The Health Research Board of Ireland, The Children's Leukaemia Research Project, The Irish Cancer Society, and The EC Biomedical programme.

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